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14. ABSTRACT

The overall objective of this Proposal is to rapidly and non-invasively assess metastatic breast cancer susceptibility to tyrosine kinase inhibitors (TKIs) by use of recombinant peptides that bind within tumors. We will then identify the receptor to which the recombinant peptide binds. We propose that this receptor protein becomes unveiled following therapy. These recombinant peptides in turn can be labeled with internal emitters to provide a means of non-invasive monitoring of tumor responsiveness to therapy. We propose that the peptide will bind to receptors within the tumor microvasculature that are specifically induced in response to therapy and are not present in untreated endothelium. These aims will test the central hypothesis that non-invasive assessment of breast tumor susceptibility to therapy can be achieved by use of recombinant peptides selected from phage-displayed libraries.

15. SUBJECT TERMS
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1. INTRODUCTION

Presently, metastatic breast cancer response is measured by assessment of tumor volumes or by repeated biopsy to analyze pharmacodynamics. These methods of monitoring breast cancer response are inefficient because volume changes typically occur after patients are on therapy for prolonged time intervals. For that reason, we have developed the hypotheses that recombinant peptides from phage-displayed peptide libraries can be selected that bind to receptors activated in response to therapy. These peptides in turn can be labeled with internal emitters to provide a means to non-invasive monitoring of responsiveness to therapy. The physiologic response to therapy is seen within 24 hours of therapy, which provides a rapid assessment using non-invasive means. The overall objective of this Proposal is to rapidly and non-invasively assess metastatic breast cancer susceptibility to tyrosine kinase inhibitors (TKIs) by use of recombinant peptides that bind within tumors. We will then identify the receptor to which the recombinant peptide binds. We propose that this receptor protein becomes unveiled following therapy. These recombinant peptides in turn can be labeled with internal emitters to provide a means of non-invasive monitoring of tumor responsiveness to therapy. We propose that the peptide will bind to receptors within the tumor microvasculature that are specifically induced in response to therapy and are not present in untreated endothelium. These aims will test the central hypothesis that non-invasive assessment of breast tumor susceptibility to therapy can be achieved by use of recombinant peptides selected from phage-displayed libraries.

2. BODY

Statement of Work (SOW) Task 1. Noninvasive assessment of metastatic breast cancer vascular response to tyrosine kinase inhibitors by use of recombinant peptides from phage-displayed libraries:

Screening of phage displayed peptide libraries have been established to discover peptide ligands that bind to tumor vasculature, cancer cells, or specific molecular targets[1-5]. Phage display technology allows for the insertion of random DNA sequences into the bacteriophage genome that in turn encode the capsid proteins, thereby providing peptides expressed on the phage surface that bind to cell surface molecules[6-7]. In our laboratory, we have isolated the HVGGSSV peptide via *in vivo* biopanning of Lewis lung carcinoma (LLC) and glioma (GL261) after treatment with radiation alone using this technology[8]. In a manuscript submitted for publication, our laboratory has shown that this peptide can detect susceptibility to treatment with tyrosine kinase inhibitors + radiation in various tumor models, including lung, brain, colon, and prostate[8]. This peptide also recognizes breast cancer cells treated with TKI alone, and this data is presented under the SOW Task 2 heading.

We have previously reported that when compared to treatment with radiation or sunitinib alone, the combination of sunitinib and radiation resulted with more cell apoptosis, improved tumor growth control and reduced tumor angiogenesis[9]. Therefore, we conducted *in vivo* biopanning to screen peptide candidates recovered from heterotopic and orthotopic gliomas (GL261) treated with sunitibib (40mg/kg) and radiation (3Gy). Sequence analysis of phage recovered from *in vivo* biopanning showed the following distribution: 1) GIRLRG–43.75%, 2) AARLY–37.5%, and 3) HMWRDSQ–18.75%. Since the GIRLRG phage is the one with the highest percent distribution in our studies, we proceeded to test whether the GIRLRG phage and peptide are able to detect treatment response in breast cancer tumor models.

Results from our laboratory have shown that peptides conjugated to near infrared imaging agents (NIR) are viable tools for rapid non-invasive imaging detection. Therefore, we have used this technique to replace our original plan to conjugate peptide to DOTA.

Sunitinib treatment elicits growth delay in mice implanted with MDA-MB-231 breast tumors.

First of all, we tested if continuous treatment of MDA-MB-231 cells with the tyrosine kinase inhibitor (TKI) sunitinib elicits tumor growth delay (**Figure 1**). We found that Sunitinib treatment is able to elicit growth delay in nude mice implanted with MDA-MB-231 tumors, and this difference is statistically significant (**Figure 1B,** P=0.001).

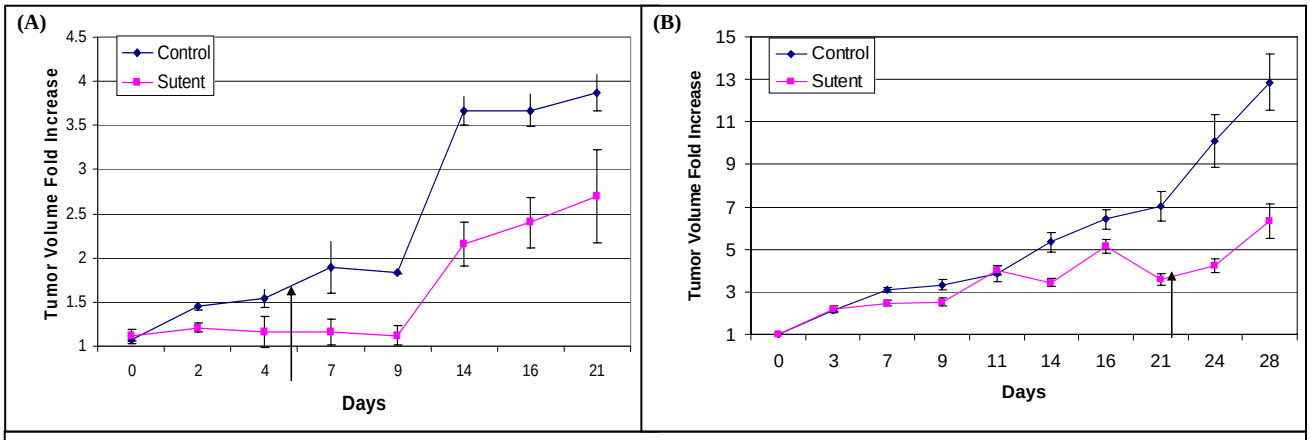


Figure 1. *Nude mice were implanted with MDA-MB231 breast cancer cells. When the tumor volume reached 0.3 cm^3, all mice were treated with either Sutent (40 mg/kg) or vehicle control daily. In panel (A), 3 control and 3 treatment mice were treated daily for 5 days and then monitored. In panel (B), 7 control and 9 treated mice were treated daily for 6 days, rest one day, and then repeated for 2 weeks. For both panels, the arrow indicates end of therapy.*

GIRLRG phage binds to Sunitinib-treated but not to untreated MDA-MB-231 breast tumors

In order to test the hypothesis that the GIRLRG phage could detect response to treatment of MDA tumor cells after they have been treated continously with Sunitinib (Figure 1), MDA-MB-231 tumor cells were injected into the hind limb of nude mice. After the tumor was successfully implanted, the mice were treated with Sunitinib daily for 6 consecutive days x 2 cycles. The Alexa-Fluor labeled GIRLRG phage was then added and we found that the GIRLRG phage binds preferentially to the treated breast tumors (**Figure 2**).

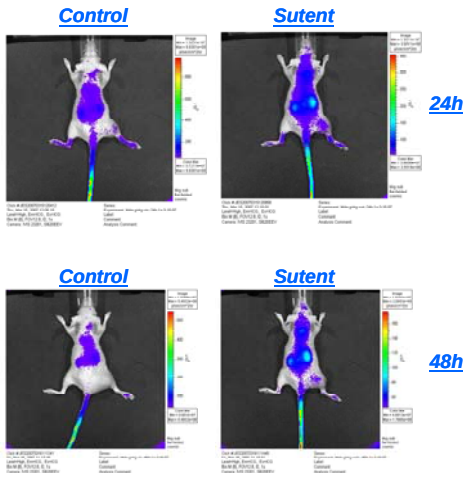


Figure 2. *Alexa Fluor-labeled GIRLRG phage binding in treated heterotopic MDA-MB231 breast tumors. MDA tumors were implanted in the hind limb of nude mice. Where indicated, mice were treated with Sunitinib (40 mg/kg) or vehicle control daily for 6 consecutive days x 3 cycles. Biodistribution of the Alexa Fluor labeled phage in tumor-bearing mice was studied with near infrared imaging at 24 hours after the addition of the first treatment with Sunitinib during the third cycle. The GIRLRG phage binds preferentially to Sunitinib-treated breast tumors that are responding to treatment compared to the untreated control.*

GIRLRG phage binds to untreated and Sunitinib-treated MCF-7 breast tumors

We also conducted a preliminary study to test whether the GIRLRG phage is able to recogonize treated MCF-7 breast cancer cells (**Figure 3**). To that effect, MCF-7 cells were implanted in the hind limbs of nude mice. Mice were treated systemically with either vehicle control or Sunitinib. The tumor in the right hind limb was irradiated with 3 Gy. Four hours after treatment, Alexa-Fluor labeled GIRLRG phage was added into the circulation and the mice were imaged with near infrared imaging at various time points. GIRLRG phage binding within treated tumors was detected within 24 hours and persisted beyond 96 hours. These preliminary studies showed strong binding in the Sunitinib-treated tumor, but also some binding in the control (untreated) tumor. Interestingly, binding was not detected in the combination of Sutent + XRT group.

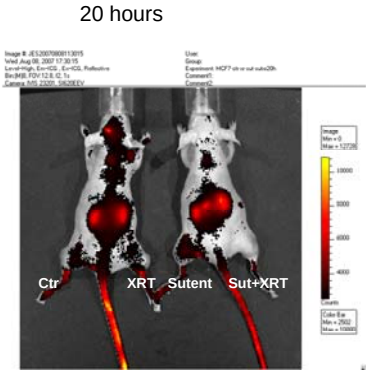
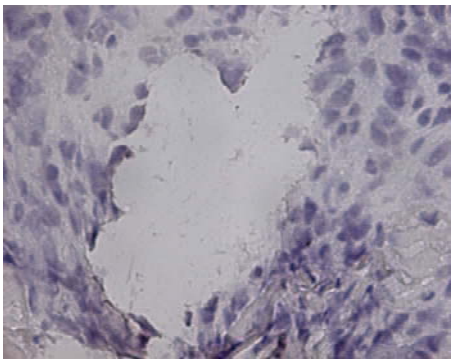


Figure 3. ***Phage GIRLRG distribution in MCF-7 treated with Sunitinib +/- XRT. Nude mice were implanted with MCF-7 breast cancer cells in both hind limbs. When the tumor volume reached 0.3 cm^3, all mice were treated once with Sutent (40 mg/kg) or vehicle control +/- 3 Gy of irradiation. Four hours after therapy, the Alexa-Fluor labeled GIRLRG phage was added. Mice were imaged at 20 hours after treatment.***

GIRLRG phage binds to treated tumor blood vessels

The phage binding within tumors was confirmed with immunohistochemical staining. Anti-phage antibody stained the GIRLRG phage within treated tumor endothelium (**Figure 4B**), but not within untreated tumors (**Figure 4A**).



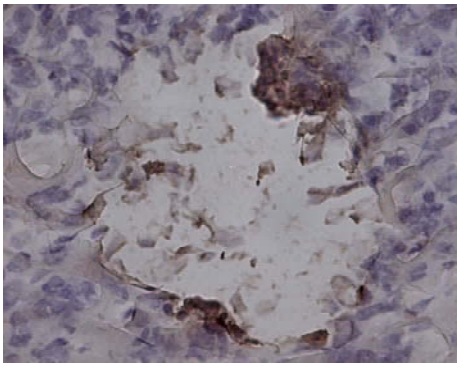


Figure 4. ***The GIRLRG phage (A, B) was administered into untreated (A) and treated (B) with radiation (3 Gy) + Sunitinib (40 mg/kg) nude mice bearing tumors. Tumors were fixed and sectioned 24 hours after addition of phage. Panel (A) shows no binding within untreated tumor. Panel (B) shows GIRLRG phage binding to the vascular endothelium of the treated tumor.***

Selection of phage-displayed peptides that bind to the microvasculature of Sunitinib-treated breast tumors

We encountered a significant delay in the approval of our Animal Care protocol, so the *in vivo* biopanning using the phage display technology to isolated peptides that could be specific to breast cancer tumor models has just been concluded. *In vivo* biopanning wsa conducted with a T7 phage-based random peptide library. Four rounds of consecutive selections were performed. The results of the peptides isolated from MCF-7 and MDA-MB-231 tumors are shown below:

Peptide Screening Results for MDA breast cancer tumors

Peptides Identified by Sequencing Analysis	Percent Distribution
EGEVGLG	58%
MRRSVGS	14%
SSAVL	8%
VLI	8%
SAGSVAL	6%
FGVR	3%
GFWEGGL	3%

*** Total number of clones assayed 36**
*** MDA heterotopic tumors were subject to 40 mg/Kg Sutent dose IP for 3 consecutive days, library injected 4 hours post-last sutent treatment, in circulation for 16 additional hours prior to peptide recovery.**
*** Four rounds of consecutive selection were performed**

Peptide Screening Results for MCF-7 breast cancer tumors

Peptides Identified by Sequencing Analysis	Percent Distribution
EGEVGLG	67%
SSAVL	18%
MRRSVGS	12%
FGVR	1%

*** Total number of clones assayed 36**
*** MCF-7heterotopic tumors were subject to 40 mg/Kg Sutent dose IP for 3 consecutive days.,**
*** First round of MDA selection library was injected 4 hours post-last sutent treatment, in circulation for 16 additional hours prior to peptide recovery**
*** Four rounds of consecutive selection were performed.**

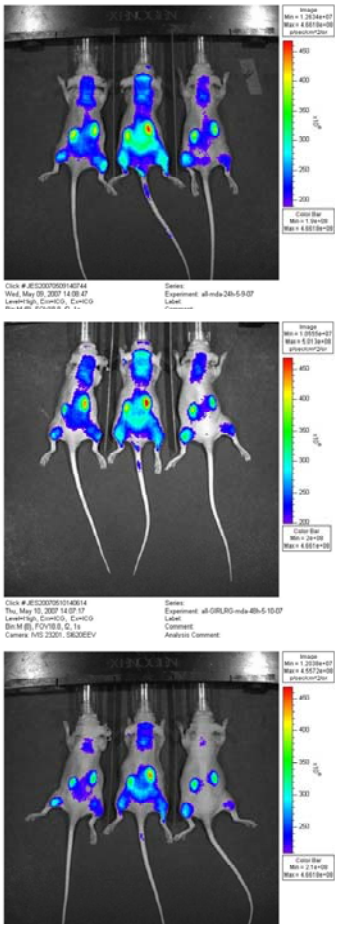
Interestingly, all four peptides isolated from the MCF-7 selection are present in the MDA selection. There are three peptides that appear to be unique to the MDA selection. We will test and prioritize these peptides during the second year of this work to determine if they can detect the susceptibility of metastatic breast cancer cells responding to treatment.

SOW Task 2. To determine whether peptide binding to treated metastatic breast cancers correlate with biological response.

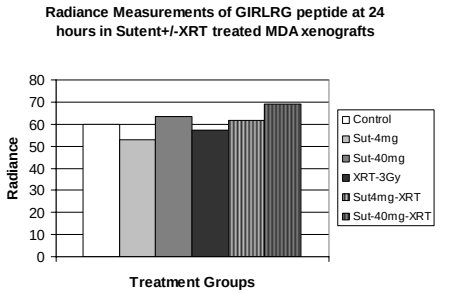
Most of the studies for this proposal this year were conducted with the GIRLRG phage and peptide. This sequence was originally isolated in our laboratory from a selection of glioma tumors, but based on our previous experience, we tested whether this sequence could also be detected in breast cancer tumor models.

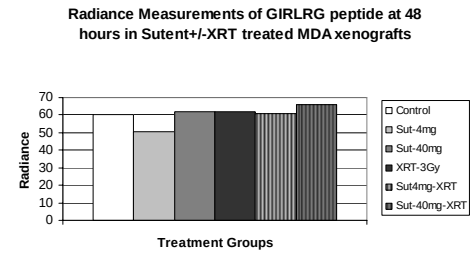
The GIRLRG peptide binds to both control and Sunitinib +/- XRT treated tumors in MDA xenografts

In order to determine if the GIRLRG peptide is the functional motif during binding to treated tumors, the GIRLRG peptide (NH2-KKGGGGIRLRG-COOH) was synthesized and labeled with Alexa-Fluor and Biotin in each of the lysines in the N-terminal group. The three glycine residues were placed at the N-terminus to separate the GIRLRG peptide from the Alexa-Fluor. Near infrared imaging was used to monitor the biodistribution of the peptide in tumor-bearing mice treated with varying doses of Sunitinib +/- radiation. We found that the GIRLRG peptide binds to both control and treated MDA tumors (**Figure 5**).



Control	+					
Sutent-40mg	_	_	+	+	_	_
Sutent-4 mg	_	_	_	_	+	+
XRT- 3 Gy	_	+	_	+	+	_





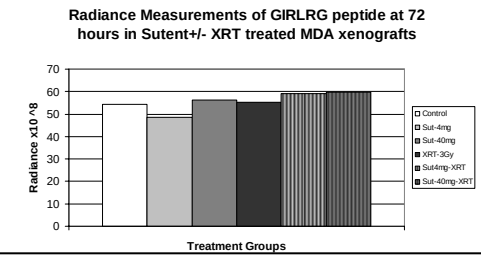


Figure 5. GIRLRG peptide binds to both control and Sunitinib +/- XRT treated tumors in MDA xenografts. Biotinilated GIRLRG peptide + Streptavidin-Alexa Fluor conjugated peptide was injected 4 hours after the first dose of treatment. Near infrared images were taken at 24h, 48h, and 72h. Radiance measurements are shown on the right.

HVGGSSV peptide can detect susceptibility of treatment response in MDA tumors

To determine whether the HVGGSSV peptide can be used to distinguish treatment response within breast cancer tumors, we studied heterotopic tumors of MDA breast cells (**Figure 6**). The HVGGSSV peptide (NH2-GGGNHVGGSSV-COOH) was synthesized and labeled with Cy7 at the N-terminal amine group. Three glycine residues were placed at the N-terminus to separate the HVGGSSV petide from Cy7. NIR imaging was used to monitor the biodistribution of the peptide in tumor-bearing mice treated with varying doses of Sunitinib +/- irradiation. Nude mice bearing MDA-MB-231 tumors were treated with 3 Gy irradiation following injection of of Sunitinib (**Figure 6A**). HVGGSSV peptide binding was not detected in untreated tumors (data not shown), whereas tumors treated with sunitinib and radiation showed near infrared imaging of peptide binding within 24 hours of injection. The treated cells showed significant increase in radiance compared to untreated controls ($p<0.05$, $n=3$). To determine whether radiance from peptide binding in tumors correlated with tumor response, we studied the dose-dependent response to sunitinib in MDA-MB-231 breast cancer cells (**Figure 6B**). MDA-MB-231 breast cancer cells were implanted into the hind limb of mice that were then treated for five consecutive days. The combination of Sunitinib with X-ray irradiation resulted in the most efficient tumor growth control, and tumors responded to high dose treatment (40 mg/kg of Sunitinib), but not 4 mg/kg, when the Sunitinib was used alone (**Figure 6B**). After the first treatment, Cy7-labeled peptide was injected into tail vein of the tumor-bearing mice, NIR images were captured, and the radiance in tumors was measured. We found a linear correlation for peptide binding (relative radiance) and tumor response (fold change in tumor volume) to treatment that is statistically significant.

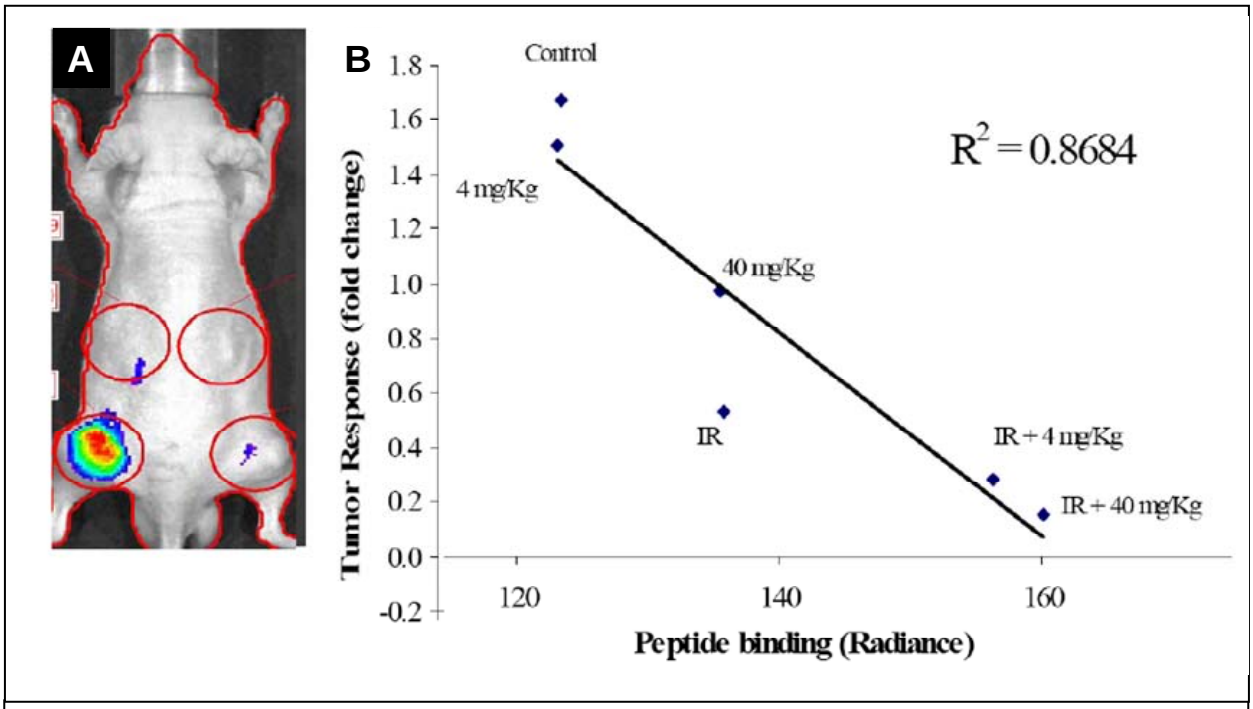


Figure 6. *HVGGSSV peptide binding detects response to therapy in a breast cancer tumor model. (A) MDA-MB-231 breast cancer cells were injected subcutaneously into the hind limb of nude mice. The tumor bearing mice were treated with sunitinib for one hour prior to irradiation. Cy7-labeled HVGGSSV peptide was injected intravenously 4 hours after treatment. Shown are NIR images obtained 48 hours after peptide injection. Irradiated tumors are indicated by arrows. (B) Shown is the linear correlation of peptide binding (relative radiance) to tumor response (tumor volume fold change) in human breast cancer MDA-MB-231 murine hind limb tumors (n=5 for each group). Radiance of peptide binding within tumors correlated with tumor growth attenuation ($R^2=0.868$; $p<0.01$).*

SOW Task 3. Identification and characterization of radiation-induced antigenic targets in metastatic breast tumor vessels.

Homology of GIRLRG peptide

BLAST search was performed for each peptide. BLAST search for the GIRLRG peptide shows that the "IRLRG" sequence is also present in the transforming growth factor alpha, which is a ligand for EGFR; the "GIRLR" sequence is also present in the fibroblast growth factor receptor 4 (FGFR4) and in thyroglobulin. For FGFR4, it has been shown that a truncated form results in pituitary tumors[10]; it interacts with N-cadherin and can cause impaired cell-matrix adhesiveness[11]; is associated with bladder cancer prognosis[12]; correlates with survival in head and neck squamous cell carcinoma[13]; and is associated with resistance to adjuvant therapy in primary breast cancer[14].

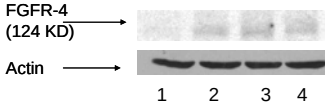
Homology of GIRLRG Peptide

- Endothelial cell growth factor 1 (platelet-derived)
- transforming growth factor, alpha, isoform CRA_e
- fibroblast growth factor receptor 4, isoform CRA_a
- Thyroglobulin
- Rho guanine nucleotide exchange factor (GEF) 11
- Monooxygenase, DBH-like 1
- protein-coupled receptor 124, isoform CRA_a
- Beta-1,4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase (N-glycosyl-oligosaccharide-glycoprotein N-acetylglucosaminyltransferase III) (N-acetylglucosaminyltransferase III) (GNT-III) (GlcNAc-T III)
- craniofacial development protein 1, isoform CRA_a
- serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 13

Levels of expression of various putative receptor candidates for GIRLRG

We subsequently conducted co-culture experiments utilizing MCF-7 grown together with HUVEC cells. We then treated the cells with Sunitinib +/- radiation, harvested them 4 hours after treatment, and then performed Western blot analyses to determine the level of expression of various of the putative candidates for receptor for GIRLRG.

Figure 7. (A) ***Co-culture experiments to determine expression of FGFR-4 with Sunitinib +/- XRT.***

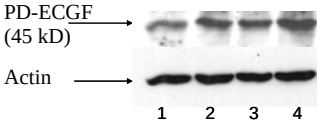


* 40 ug/ lane, 1:500 primary, 1:5000 secondary

1. Huvec+MCF-7 Control
2. Huvec+MCF-7 Sutent (5uM)
3. Huvec+MCF-7 XRT (3Gy)
4. Huvec+MCF-7 Sutent+XRT

Results:
In co-culture system with MCF-7 cells FGFR4 appears to increase with Treatment groups compared to control

Figure 7. (B) ***Co-culture experiments to determine expression of PD-ECGF with Sunitinib +/- XRT.***



* 40 ug/ lane, 1:500 primary, 1:5000 secondary

1. Huvec+MCF-7 Control
2. Huvec+MCF-7 Sutent (5uM)
3. Huvec+MCF-7 XRT (3Gy)
4. Huvec+MCF-7 Sutent+XRT

Results:
In co-culture system with MCF-7 cells PD-ECGF appears to increase with Treatment groups.

Additional efforts to continue to identify potential targets and to isolate the receptor have been initiated. Thus, affinity purification using HUVEC co-cultures with breast cancer cells are being investigated.

3. KEY RESEARCH ACCOMPLISHMENTS

- HVGGSSV peptide can detect susceptibility of treatment response in MDA tumors.
- GIRLRG phage binds preferentially to MDA tumors treated continuously with Sunitinib compared to untreated control.
- GIRLRG peptide binds to both untreated and single dose Sunitinib-treated breast cancer tumors.
- We have identified four peptide sequences (EGEVGLG, SSAVL, MRRSVGS, and FGVR) that have potential to elicit binding to Sunitinib-treated breast tumors.

4. REPORTABLE OUTCOMES

Han Z, Fu A, **Diaz R**, Wang H, Geng L, and Hallahan DE. Rapid Assessment of Cancer Susceptibility to Molecular Targeted Therapy by Use of Peptide Imaging Agents. *Submitted for Publication.*

Huamani J, Passarella RJ, Onishko HM, Fu A, Geng L, Han Z, Hallahan DE, and **Diaz R** (2007). Rapid Assessment of Malignant Glioma Susceptibility to Molecular Targeted Therapy. *Poster and Poster Discussion, ASTRO 49th Annual Meeting.*

Fu A, **Diaz R**, Onishko H, Huamani J, and Hallahan DE (2007). Non-invasive Assessment of Cancer Susceptibility to Therapy. *Poster and Poster Discussion, ASTRO 49th Annual Meeting*.

Diaz R, Huamani J, Fu A, Han Z, Wang H, Geng L, and Hallahan DE (2007). Imaging Cancer Response to Therapy. *Vanderbilt-Ingram Cancer Center (VICC) Retreat.*

Diaz R, Huamani J, Fu A, Han Z, Wang H, Geng L, and Hallahan DE (2007). Imaging Cancer Response to Therapy. *Vanderbilt University Medical Center's 25th Annual Research Forum.*

Diaz R, Huamani J, Fu A, Han Z, and Hallahan DE (2007). Recombinant Peptides as Biomarkers for Cancer Response to Tyrosine Kinase Inhibitors Combined with Radiation. *Press Conference, Personalized Medicine Session, American Association for Cancer Research (AACR) Annual Meeting.*

Diaz R, Huamani J, Fu A, Han Z, and Hallahan DE (2007). Recombinant Peptides as Biomarkers for Cancer Response to Tyrosine Kinase Inhibitors Combined with Radiation. *AACR Annual Meeting.*

Diaz R, Huamani J, Fu A, Han Z, and Hallahan DE (2007). Non-invasive Imaging of Cancer Response to Treatment Using Recombinant Peptides. *Poster and Oral Presentation, AACR-ACS 1st Joint Conference, Chemistry in Cancer Research: A Vital Partnership.*

5. CONCLUSION

We have identified peptides, like HVGGSSV, that bind to breast tumors treated with Sunitinib, and that the level of binding correlates with tumor susceptibility to therapy. Additionally, the GIRLRG phage elicits differential binding to MDA and MCF-7 cells and therefore warrants further investigation as to what is the putative receptor that is present even in untreated MCF-7 cells. This could open the possibility of utilizing this peptide for potential targeted drug delivery systems.

Furthermore, the identification of novel peptides (EGEVGLG, SSAVL, MRRSVGS, and FGVR) isolated specifically from both breast cancer tumor cells (MCF-7 and MDA) treated with Sunitinib shows potential for further investigation and validation of these peptides/phage with additional response to other receptor tyrosine kinase inhibitors. In addition, we would also like to prioritize these peptides based on their ability to discern treatment response within metastatic breast tumors.

Therefore, phage display technology promises to aid the discovery of useful imaging peptides that facilitate molecular imaging of breast cancer pharmacodynamic response to therapy with tyrosine kinase inhibitors. We have shown that this technology can be used to select phage displayed peptides that distinguish treated from untreated breast tumors. These peptides can serve as "molecular beacons" that bind to susceptible cancers, allowing for a more rapid assessment of cancer responsiveness to therapy. Thus, rapid noninvasive assessment of pharmacodynamic response of breast cancer promises to minimize the duration of ineffective treatment regimens in cancer patients and speed drug development.

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7. APPENDICES

None included.